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(54) A mutant strain of *Clostridium thermoaceticum* useful for the preparation of acetic acid.

(57) A biologically pure mutant of *C. thermoaceticum* (ATCC No. 39,289) useful for the production of acetic acid by a fermentation reaction is disclosed. This mutant is capable of growing at a pH below 5.0 and shows a specific growth rate of at least 0.30 hr⁻¹ when continuously cultured under optimum conditions.

regulates
remaining in culture
at const. level

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A MUTANT STRAIN OF CLOSTRIDIUM
THERMOACETICUM USEFUL FOR THE
PREPARATION OF ACETIC ACID

This invention relates to a novel mutant strain of Clostridium thermoaceticum and to its use in the preparation of acetic acid.

The production of organic chemicals by microorganisms is well known to those familiar with the fermentation art. Such fermentation reactions frequently produce a variety of products in dilute aqueous solutions. The expense of separating the chemicals from each other and from the large volumes of water has been so great that production of chemicals by fermentation has not been able to compete with the production of the same chemicals from fossil fuel sources. However, the gradual depletion of petroleum fossil fuel with a resultant increase in prices of petrochemical feedstocks has revived interest in fermentation reactions which can convert renewable raw materials into simple organic chemicals.

1 Fermentations that produce a single product are
2 particularly desirable since product isolation from such
3 reactions is simplified. Certain microorganisms, known as
4 homoacidogens, can be used in such a procedure to give a
5 single acid when grown on a variety of hexoses, pentoses
6 and lactic acid. The fermentation of a hexose such as glucose
7 by Clostridium thermoaceticum, hereafter abbreviated C.
8 thermoaceticum, is especially attractive since it can
9 produce theoretically 3 moles of acetic acid from 1 mole of
10 the sugar.

11
12 C. thermoaceticum was first isolated by Fontaine,
13 et al, J. Bacteriol., 43, 701-715 (1942). The wild strain
14 is an anaerobe that grows best at a pH of about 7. Its
15 growth is inhibited by low pH, acetic acid, and
16 acetate. For these reasons, this strain does not produce
17 acetic acid in high concentration.

18
19 Various workers have attempted to obtain mutant
20 strains of C. thermoaceticum that would produce higher
21 concentrations of acetic acid in a fermentation reaction.
22 Wang, et al, AIChE Symp. Ser. 181, 74, 105-110 (1978),
23 described an improved strain of the microorganism that had
24 a higher tolerance for sodium acetate. Schwartz and Keller,
25 Applied and Environmental Microbiology, 43, 117-123 (1982),
26 reported isolation of a strain of C. thermoaceticum capable
27 of growing and producing acetic acid at a pH of 4.5. This
28
29
30

work is also disclosed in U.S. Patent 4,371,619.

We have now isolated a new mutant of C. thermoaceticum; capable of growing at a pH of less than 5, which is a more efficient producer of acetic acid than any of the strains previously reported.

In accordance with the present invention, there is provided a biologically pure culture of a mutant strain of the microorganism C. thermoaceticum, useful for the production of acetic acid when grown in an aqueous medium containing assimilable sources of carbon, nitrogen and inorganic substances. This culture is capable of growing at a pH below 5.0 at a temperature of from 50°C to 65°C. It also has a specific growth rate of at least about 0.30 hr^{-1} when grown in a continuous culture at pH 7 and 58°C. The strain is deposited at the American Type Culture Collection as ATCC No. 39,289.

Also provided in accordance with this invention is a method for converting carbohydrates to acetic acid by growing C. thermoaceticum in an anaerobic fermentor on an aqueous medium containing assimilable sources of carbon, nitrogen and inorganic substances, in a pH controlled continuous fermentation at a pH of about 5 to about 8, at a temperature of about 50°C to about 65°C and at a dilution

1 rate of about 0.01 to about 0.40 per hour, using the strain of
2 C. thermoaceticum, ATCC No. 39,289.

3
4
5
6 The parent culture used for the development of the
7 mutant strain of this invention was a wild type of C.
8 thermoaceticum, DSM 521. The cultures used were obtained
9 from the Massachusetts Institute of Technology, whose workers
0 in turn had obtained it from Dr. H. G. Wood, Case Western
1 Reserve University, Cleveland, Ohio.

2
3 Growth was monitored by measuring the absorbance
4 at 540 nm. A Spectronic 20 spectrophotometer (Bausch &
5 Lomb, Inc., Rochester, New York) was used for the
6 measurements. The sample was diluted with distilled water
7 to read between 0.1 and 0.7 absorbance and the measurements
8 were made in matched Hungate tubes of 16 mm outside diameter
9 against a distilled water blank. Two or three grains of
0 sodium dithionite were added to the diluted sample prior
1 to reading when resazurin indicator was used to maintain
2 it in its colorless state.

3
4 Acetic acid and glucose concentrations were
5 determined using high-performance liquid chromatography (HPLC).
6 A sample of fermentation mixture was centrifuged at about
7 10,000 x g.
8
9

Claims
use
calculations
to detect
glucose
level
ratio

for 10 minutes to pellet the cells. Components of the supernatant were chromatographed by elution with 0.06 N H_2SO_4 from a cation-exchange resin in the hydrogen form. The eluted components were detected by means of a differential refractometer, plotted on a recorder and quantitated using an electronic integrator. The area underneath the curve which represents concentration of each component was reported as a percentage of the total area. The general procedure is that given in "Analyses of Carbohydrate Mixtures by Liquid Chromatography", Am. Soc. Brew. Chem. Proc., 1973, pp. 43-46. The separations were made on a 1-foot HPX-87 column in the hydrogen form, available from Bio-Rad Laboratories, Richmond, California.

In the description of this invention, the words "dilution rate", as used in this application, have the dimensions of per hour (hr^{-1}). This rate is obtained by dividing the flow rate of the medium in volume units per hour by the operating volume of the reactor.

The term "specific growth rate", as used herein, represents the rate of growth per unit amount of biomass in the fermentation reactor. It also has the dimension of reciprocal time (hr^{-1}). When a continuous fermentation vessel is operating at steady state conditions, the amount of biomass in the culture is constant and the specific growth rate equals the dilution rate.

1 The maximum specific growth rate was determined by
2 the method described in Pirt, S. J., "Principles of Microbe
3 and Cell Cultivation", John Wiley & Sons, New York, 1975,
4 p. 33. This was accomplished by increasing the flow rate
5 in the chemostat above the rate at which washout of the
6 cells occurs. Absorbance readings were measured at
7 various times and the natural logarithms of the optical
8 density readings were plotted against time. The slope of
9 this line plus the dilution rate at which washout occurred
10 represents the maximum specific growth rate for the
11 microorganism. The mass doubling time for the cells, also
12 called the generation time, is calculated by dividing $\ln 2$
13 by the maximum specific growth rate.

14
15 The parent organism was grown in a chemostat
16 using conditions of continuous culture in a medium in which
17 the pH was gradually lowered over a period of time. The
18 operation of such a chemostat and its use for obtaining
19 spontaneous mutations of bacteria growing in it was
20 described by Novick, A. and Szillard, L., Science, 112,
21 715-716 (1950). The chemostat operation used the principle
22 of biomass feedback in which the amount of nutrient fed to
23 the growing culture was controlled by the pH of the medium.
24 This technique is designed to select for genetically
25 different organisms, in this case those growing more
26 rapidly at lower pH. Using this technique, a mutant strain
27 was obtained which would grow in a medium having a pH of
28
29

feed rate
controlled
by pH

5.0 or below. This mutant strain is distinctly different from the parent strain of C. thermoaceticum, which shows little growth in a medium with a pH below about 6.4.

The mutant strain of the microorganism described herein is a new composition of matter. It has been deposited in the American Type Culture Collection in Rockville, Maryland, and will be maintained in that depository during the life time of this patent. It is available as ATCC No. 39,289.

The mutant strain of this invention is a thermophilic anaerobe that is grown in a medium and under an atmosphere from which oxygen has been excluded. It is capable of growing in an aqueous medium containing assimilable sources of carbon, nitrogen and inorganic substances at a pH below 5.0 to produce acetic acid. Preferred carbon sources are glucose and fructose. Although growth and acetic acid production occur slowly at a pH below 5.0, they occur more rapidly at higher pH with a preferred pH being between about 6.9 and 7.4. Growth of the microorganism occurs at a temperature from about 50°C to about 65°C with a preferred temperature being in the range of about 56°C to about 60°C. The mutant strain is capable of growing with a specific growth rate of at least about 0.30 hr⁻¹ at pH 7 and 58°C.

1 The mutant strain of this invention is useful
2 for converting carbohydrates to acetic acid when it is
3 grown in an anaerobic fermentor in a pH controlled
4 continuous fermentation. Such a fermentation can be carried
5 out at a dilution rate of from 0.01 to 0.4 per hour when
6 the pH is controlled in a range of from 5 to 8 and the
7 temperature is held in the range of from 50°C to 65°C.
8

9 The present mutant strain differs from the parent
10 in its ability to grow in a medium at pH 5. In addition,
11 it shows a maximum specific growth rate about 50% greater
12 than that of the parent strain when both are grown in a
13 medium at pH 7.
14

15 The mutant strain and process of this invention
16 are further illustrated by the following examples:

18 EXAMPLE 1

19
20 The parent strain of the microorganism, C.
21 thermoaceticum (DSM 521), was kindly furnished to us by
22 workers at the Massachusetts Institute of Technology,
23 who in turn had obtained it from Dr. H. G. Wood,
24 Case Western Reserve University, Cleveland, Ohio. It is
25 available from the Deutsche Sammlung von Mikroorganismen
26 in Göttingen, West Germany, as DSM 521.

*Suggests
an alkaline
pH limit
is low*

Medium preparation and cultivation of samples were carried out using standard anaerobic techniques as described by Hungate, R. E., "A Roll Tube Method for Cultivation of Strict Anaerobes", in Methods and Microbiology, edited by J. R. Norris and D. W. Ribbons, Vol. 3B, Academic Press, New York, 1969, pp. 117-132, and by Miller and Wolin, Appl. Microbiol., 27, 985 (1974).

The medium used for growth of the organism had the following composition:

GROWTH MEDIUM

<u>Component</u>	<u>Concentration (g/liter)</u>
A. Glucose	30.0
B. NaHCO ₃	16.8
K ₂ HPO ₄	7.0
KH ₂ PO ₄	5.5
C. Yeast Extract	5.0
Tryptone	5.0
(NH ₄) ₂ SO ₄	1.0
MgSO ₄ ·7H ₂ O	0.25
Fe(NH ₄) ₂ (SO ₄) ₂ ·6H ₂ O	0.04
Co(NO ₃) ₂ ·6H ₂ O	0.03
NaMoO ₄ ·2H ₂ O	0.0024
Resazurin (0.20 g/100 ml solution)	1.0 ml/l

Solutions of components of Groups A, B, and C were sterilized separately before combining to make a

medium of the given composition. Then 0.5 g of sodium thioglycolate, 5.6 mg of nicotinic acid and 1 ml of a trace salt solution were added per liter of medium. The trace salt solution had the following composition:

TRACE SALT SOLUTION

<u>Component</u>	<u>Concentration (g/liter)</u>
Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate	5.00
MnCl ₂ ·4H ₂ O	5.00
Na ₂ SeO ₃	0.20
H ₂ BO ₃	0.100
ZnCl ₂	0.050
AlK(SO ₄) ₂ ·12H ₂ O	0.100
NiCl ₂ ·6H ₂ O	0.020
CuCl ₂ ·2H ₂ O	0.010

A continuous fermentation was carried out in a 1-liter Bellco fermentor (Bellco Glass Inc., Vineland, New Jersey). This fermentor was fitted with an inlet tube for medium addition, an overflow outlet tube, a magnetically driven agitator and an Ingold pH probe (Ingold Electrodes Inc., Andover, Massachusetts). The pH probe was attached to a Chemtrix type 45 AR pH controller (Chemtrix Inc., Hillsboro, Oregon). The pH controller activated peristaltic pumps which added medium and removed effluent from the fermentor whenever the pH reached a low-control set point. This arrangement required that the culture generate sufficient

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acetic acid to lower the pH to the control point before fresh medium was added to the fermentor to raise the pH back above the control point. The level of medium in the fermentor was kept constant by the overflow effluent pump system and the fermentor was constantly mixed and sparged with pure CO₂ at a slight positive pressure (2 to 3 cm H₂O). The temperature was maintained at 58 ± 1°C by means of heated propylene glycol which circulated through the jacket of the fermentor.

The original culture was grown in a medium with the pH set at 6.3. As the organism grew, selection for faster-growing organisms occurred and the rate of medium delivery increased. Once a stable near-equilibrium condition was reached, the pH control point was lowered about 0.1 pH unit. Selection was then continued at this lower pH until a new equilibrium condition was reached. Following this procedure, it was possible to obtain a culture of C. thermoaceticum growing at or below a pH of 5.0. A discrete colony of this new mutant was isolated, replicated and grown in fresh medium. The cells were stored by freezing in glycerine or by lyophilization. This strain is available from the American Type Culture Collection as ATCC No. 39,289.

✓ pH 4 add
meat
glu

pH 7 = sub
main add

pH = 6.3
pH = sub add

when pH
add
nutrient

Although the strain grows at pH 5 or slightly below, the specific growth rate at this pH is quite small. The maximum observed specific growth rate at pH 5 was 0.023 hr^{-1} , corresponding to a generation time of 30 hours. The product concentration in a continuous reactor operated under these conditions averaged about 10 g/l. However, when this strain was grown in a chemostat at a neutral pH, a much improved growth rate was observed as noted in the following example.

EXAMPLE 2

The low pH tolerant strain of C. thermoaceticum isolated in Example 1 was grown in a chemostat held at a pH of 7.0 and at a temperature of $58 \pm 1^\circ\text{C}$. The equipment and operation of the chemostat was similar to that given in Example 1 except that a constant delivery feed pump was used in place of the pH control pump for supplying medium to the fermentor. Fermentor volume was kept constant by use of an effluent tube positioned at the desired level. Steady state growth rates of from 0.25 hr^{-1} to 0.31 hr^{-1} were observed. The maximum specific growth rate was determined to be 0.33 hr^{-1} which corresponds to a mass doubling time of 2.1 hours. This compares with a maximum specific growth rate of 0.22 hr^{-1} for the parent culture as reported by Wang, et al, AIChE Symp. Ser. 181, 74, 105-110 (1978).

0191
+ alkali
sugars
admix
alkali
200m
pH 7.0
100m

EXAMPLE 3

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The low pH tolerant strain of C. thermoaceticum isolated in Example 1 was compared with the parent strain in these further experiments. Both strains were grown to log phase before 0.3-ml samples were used to inoculate 10 ml of growth medium in 18-mm, aluminum-sealed, Bellco tubes (Bellco Glass, Inc., Vineland, New Jersey). The growth medium was of the same concentration as that used in Example 1 except that the concentration of glucose was 2% and the buffer salts (Group B of the medium) were only half as concentrated. The pH of the medium was adjusted with acetic acid. The tubes were incubated at 58°C with CO₂ in the headspace. When the initial pH of the medium was 5.5, only the mutant strain of this invention showed growth as measured by increase in absorbance readings. After an initial lag period of about 40 hours, it grew at a specific growth rate of 0.063 hr⁻¹. In contrast, the parent strain showed no growth even after incubation for 120 hours.

When the initial pH of the medium was 6.1, the mutant strain of this invention exhibited a specific growth rate of 0.099 hr⁻¹ with almost no lag period. The parent strain, after an initial lag period of about 10 hours, grew at a specific growth rate of 0.069 hr⁻¹.

1 Thus, it is apparent that there has been provided,
2 in accordance with the invention, a strain of C.
3 thermoaceticum useful for the production of acetic acid
4 which is superior to the strains of the prior art. While
5 the invention has been described in conjunction with
6 specific embodiments thereof, it is evident that many
7 alternatives, modifications, and variations will be
8 apparent to those skilled in the art in light of the
9 foregoing description. Accordingly, it is intended to
0 include all such alternatives, modifications, and
1 variations as set forth within the spirit and scope of
2 the appended claims.

CLAIMS

1. A biologically pure culture of a mutant strain of the microorganism C. thermoaceticum, useful for the production of acetic acid when grown in an aqueous medium containing assimilable sources of carbon, nitrogen and inorganic substances, characterized in that said mutant strain is capable of growing at a pH below 5.0 at a temperature of from 50°C to 65°C, has a specific growth rate of at least about 0.30 hr⁻¹ when grown in a continuous culture at pH 7 and 58°C, and further characterized as ATCC No. 39,289.

2. A method for converting carbohydrates to acetic acid by growing C. thermoaceticum in an anaerobic fermentor on an aqueous medium containing assimilable sources of carbon, nitrogen and inorganic substances, in a pH controlled continuous fermentation at a pH of about 5 to about 8, at a temperature of about 50°C to about 65°C and at a dilution rate of about 0.01 to about 0.40 per hour, characterized in that the strain of C. thermoaceticum used in the process is the strain of claim 1.

3. The method of claim 2 characterized in that the assimilable source of carbon is selected from the group consisting of glucose, fructose, and mixtures of glucose and fructose.

4. The method of claim 2 characterized in that the fermentation is carried out at a pH between about 6.9 and 7.4.

5. The method of claim 4 characterized in that the pH of the fermentation is controlled at a pH of about 7.

6. The method of claim 2 characterized in that the fermentation is carried out at a temperature between about 56°C and about 60°C.

7. The method of claim 6 characterized in that the temperature is controlled at $58 \pm 1^\circ\text{C}$.

8. The method of claim 7 characterized in that the pH of the fermentation is controlled at a pH of about 7.

9. The method of claim 8 characterized in that the assimilable source of carbon is selected from the group consisting of glucose, fructose and mixtures of glucose and fructose.



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**DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4,
OF THE EUROPEAN PATENT CONVENTION**

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits: ATCC No. 39,289